Materials and Methods

Coronavirus phylogenetic analysis. Phylogenetic trees were constructed using the Geneious Tree Builder in Geneious 9.1.3 (http://www.geneious.com). Trees and protein sequence identities were exported to Evolview (www.evolgenius.info/evolview) for tree visualization, customization and sequence identity heat map creation. Accession numbers utilized for phylogenetic analysis are as follows: PEDV (NC_003436), HCoV-229E (JX503060), HCoV-NL63 (JX504050), PoDelta-CoV (KR265863), AIBV (NC_001451), HCoV-OC43 (AY903460), HCoV-HKU1 (DQ415904), BtCoV-HKU5 (NC_009020), MERS-CoV (JX869059), SARS-CoV (AY278741), BtSCoV-SHC014 (KC881005), BtSCoV-WIV1 (KF367457), BtCoV-HKU3 (DQ22305), BtCoV-HKU9 (EF065516).

Determination of genomic viral RNA, subgenomic viral RNA and cell death factor gene expression via qRT-PCR. Total RNA from HAE cells was harvested in Trizol and isolated using the Zymo Direct-zol RNA MiniPrep Kit (Zymo Research Corp.). First-strand cDNA was generated using Superscript III (Life Technologies). For quantification of viral RNA, real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) on a Roche LightCycler 480 (Roche) using primer sets for genomic (ORF1) or subgenomic RNA (ORFN) (Table S1). Results were normalized to GAPDH and analyzed using the ΔΔCt method. Quantitation of apoptosis factor gene expression by qRT-PCR was performed using commercially validated TaqMan-based primer-probe sets (Table S2) and TaqMan Universal PCR Mix (Life Technologies). Results were then normalized as described above.

***In vitro* metabolism of GS-5734 in 2B4 cells.** 2B4 cells were plated at a density of 0.40 X 10^6 cells/well in a 12-well plate. 24hr later, cell culture media was replaced. At 48hr post-plating, media was replaced with media containing 1μM GS-5734 and incubated for 48hr at 37 ˚C. At 2, 8, 24, 36 and 48hr post addition, medium was aspirated, cells were washed twice with 2 mL of ice-cold 0.9% normal saline, scraped into 0.5 mL ice-cold 70% methanol containing 100 nM 2-chloro-adenosine-5'-

triphosphate (Sigma-Aldrich) as an internal standard and stored at -20°C. Extracts were centrifuged at 15,000 x *g* for 15 minutes and supernatants were transferred to clean tubes for evaporation in a MiVac Duo concentrator (Genevac). Dried samples were reconstituted in mobile phase A containing 3 mM ammonium formate (pH 5) with 10 mM dimethylhexylamine (DMH) in water for analysis by LC-MS/MS, using a multi-stage linear gradient from 10% to 50% acetonitrile in mobile phase A at a flow rate of 150 μL/min. Analytes were separated using a 50 x 2 mm, 2.5 μm Luna C18(2) HST column (Phenomenex) connected to a LC-20ADXR (Shimadzu) ternary pump system and HTS PAL autosampler (LEAP Technologies). Detection was performed on an API 5000 MS/MS (Applied Biosystems) operating in positive ion and multiple reaction monitoring modes. Analytes were quantified using a 7-point standard curve ranging in concentration from 0.274 to 200 pmol prepared in extracts from untreated cells.

Cytotoxicity and determination of the half-life of nucleotide triphosphate (TP) in primary normal human bronchiolar epithelial (NHBE) cells. Normal human bronchial epithelial (NHBE) cells (Lonza, #CC-2540, Donor 29132) were cultured in Bronchial Epithelial Growth Media (BEGM) (Lonza, #CC-3170). For CC_{50}, NHBE were plated at 10,000 cells/well in a 96-well plate and incubated at 37°C. 24hr post plating, three-fold serially diluted compound was added in triplicate (final DMSO 1%) and incubated at 37˚C for 48hr after which viability was measured via CellTiter-Glo (Promega) assay quantitated on an Envision plate reader (PerkinElmer). Percent viability values were determined by normalization to 1.0% DMSO-only control wells with background luminescent signal subtracted out. For half-life determination, NHBE cells were seeded at 0.2 X 10^6 cells/well and were incubated with GS-5734 at 1 μM for 2hr, after which the media was removed, cells were washed twice and incubated with fresh medium without compound additional 30 hr. At 2, 4, 8, 24 and 32 hr post-

removal of drug, cells were harvested and processed as described above for the *in vitro* metabolism of GS-5734 in 2B4 cells.

Stability of GS-5734 in WT or *Ces1c*$^{-/-}$ mouse plasma. Duplicate samples of plasma from either wild-type C57BL/6 or esterase-deficient (*Ces1c*$^{-/-}$) C57BL/6 mice were incubated with GS-5734 (1μM final) at 37˚C. At 5, 10, 30 and 60 min post GS-5734 addition, 25 µL was removed and quenched with 100 μL of a 90% methanol:acetonitrile mixture (1:1, v:v) with 10% water containing 20 nM 5-(2-Aminopropyl)indole (5-IT; Internal Standard). The reference at 0 min was obtained through immediate quenching following the addition of GS-5734. 100 μL of the quenched mixture was then filtered (Agilent Captiva 96 0.2μm) and dried under a stream of nitrogen. After reconstitution in a mixture of 1% acetonitrile and 99% water containing 0.01% formic acid, a 10 μL aliquot was injected onto a Waters Xevo TQ-S mass spectrometer for analysis. The percent GS-5734 remaining at each time point, relative to the reference, was reported. Ces1c enzymatic activity was confirmed using positive control compound tenofovir alafenamide (TAF).

Quantitation of nucleotide species in the lung following GS-5734 administration in *Ces1c*$^{-/-}$ mice. Mice were dosed with 50 mg/kg GS-5734 subcutaneously once daily for seven days. 4 hr after the last injection, lungs from six mice were harvested and snap frozen and processed and analyzed as noted in the main Materials and Methods to obtain the concentration of lung nucleotide monophosphate (Nuc-MP), diphosphate (Nuc-DP) and triphosphate (Nuc-TP).

Assessment of mouse lung pathology and virus lung antigen staining. After at least 7 days in 10% buffered formalin, 5μm tissue sections were prepared from paraffin embedded tissue by the UNC Animal Histopathology Core. Parallel sections were either stained with hematoxylin and eosin or for viral antigen as described(*37*). Blinded slides were assessed for virus associated lung pathology as well

as location and prevalence of viral antigen. Images were captured at both 10 and 40X using an Olympus BX41 microscope equipped with an Olympus DP71 camera.

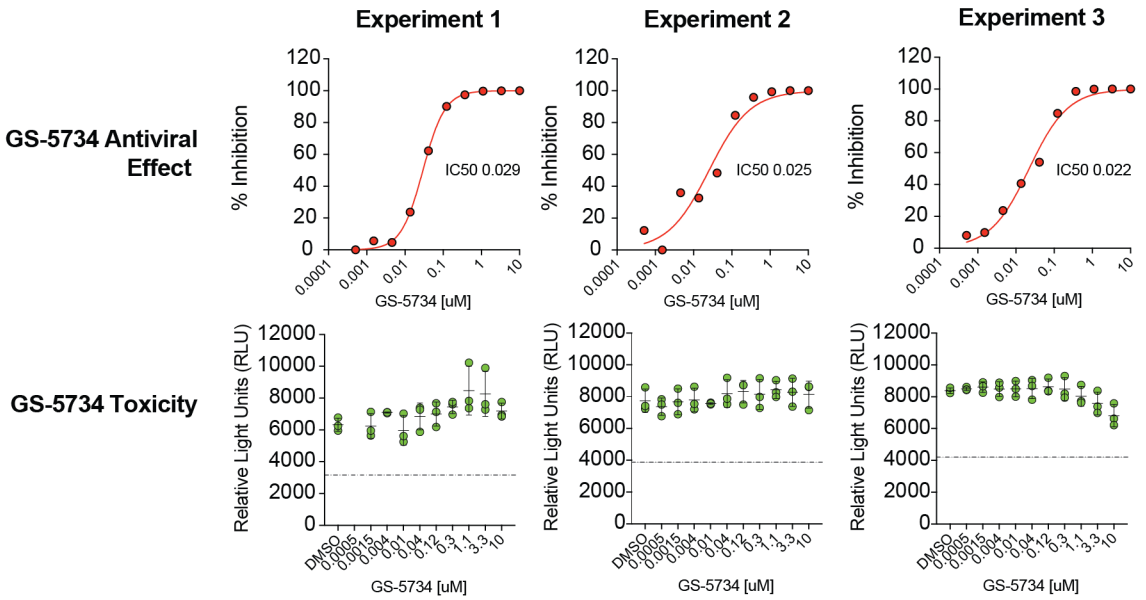


Figure S1: In vitro toxicity and efficacy of GS-5734 in Calu-3 2B4 cells. (Top) Mean percent inhibition of MERS-CoV replication by GS-5734. 2B4 cells were infected in triplicate with MERS-CoV nLuc at an MOI of 0.08 in the presence of a varying concentrations of GS-5734 for 48hr after which replication was measured through quantitation of MERS-CoV expressed nano luciferase (nLUC). **(Bottom)** Cytotoxicity in 2B4 cells treated similarly to those above. Viability was measured via CellTiter-Glo. The dotted line for each toxicity plot represents the CC_{50} value.

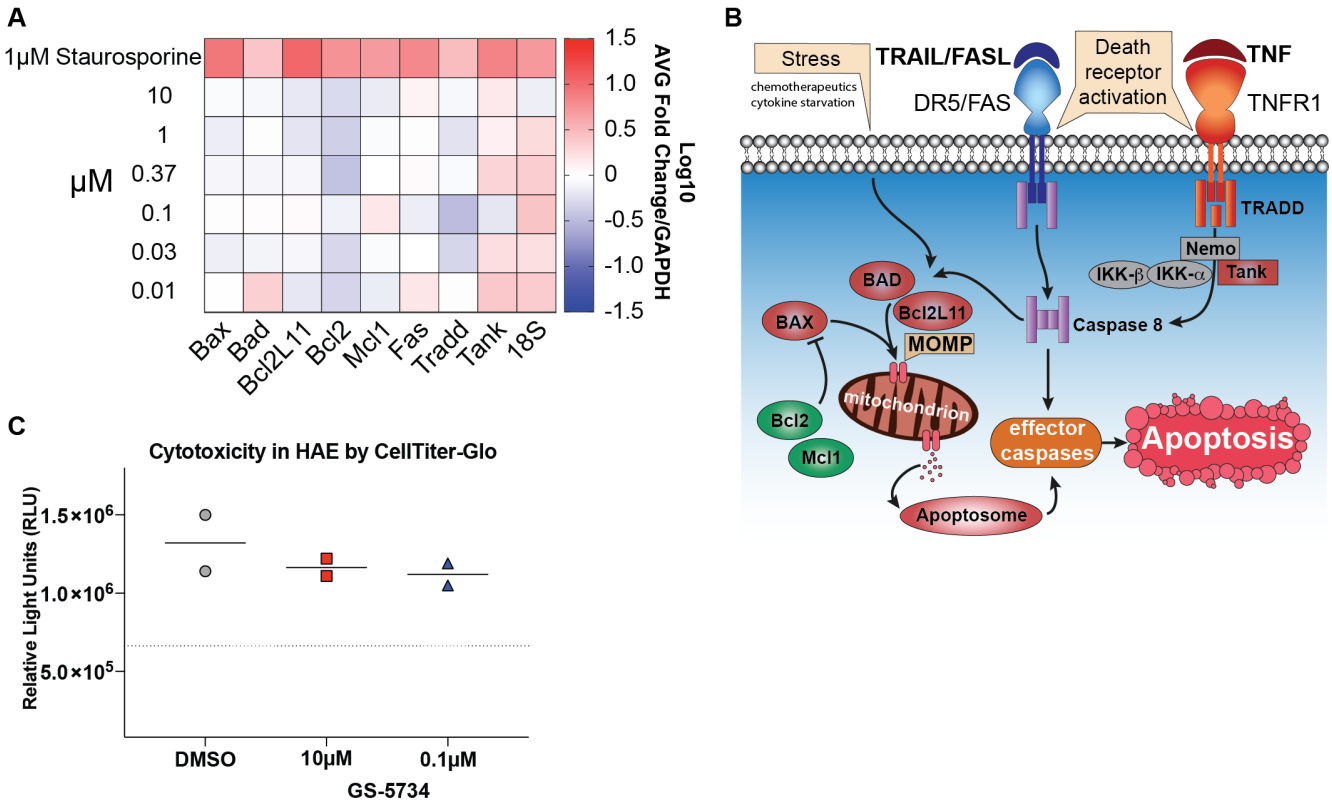
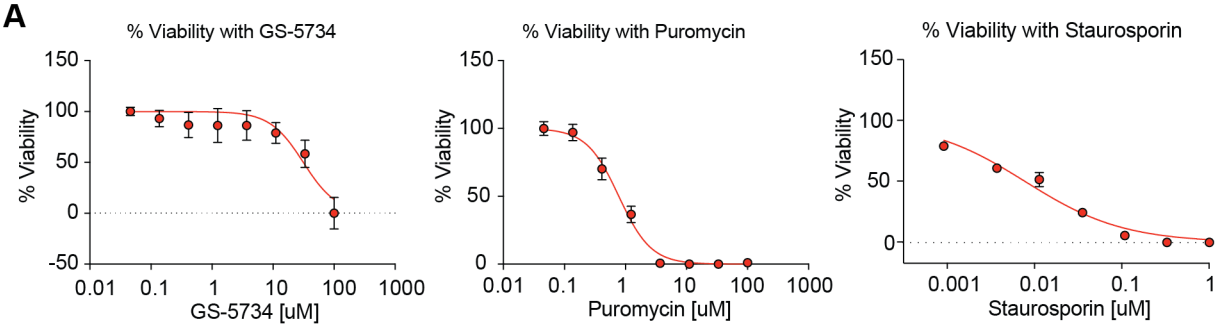


Figure S2: In vitro toxicity of GS-5734 in primary human airway epithelial (HAE) cell cultures. (A) Heatmap of death factor gene expression displaying the average LOG_{10} fold-change from GAPDH. HAE cell cultures treated with varying concentrations of GS-5734 or 1μM Staurosporine in duplicate. qRT-PCR was performed on total RNA 48hr post treatment with primer sets directed against cell death factors from multiple death pathways or house-keeping genes. Similar data was obtained in two separate experiments. (**B)** Cartoon demonstrating the role of molecules queried in Fig. S2A in multiple aspects/pathways of cell death. Pro-apoptotic molecules are labeled with red, purple and blue while anti-apoptotic molecules are labeled with green shapes. (**C)** CellTiter-Glo assay on GS-5734 treated HAE cell cultures. Duplicate HAE cultures were treated with 10 or 0.1μM GS-5734 or DMSO. 48hr post-treatment, toxicity was assessed via CellTiter-Glo assay. Per data group, the mean is indicated with a line. The CC_{50} threshold is indicated by the dotted line.

A



B

	CC50 (µM)			Summary Statistics		
Compound	**Exp 1**	**Exp 2**	**Exp 3**	**Avg CC50**	**Standard Dev**	**CV%**
GS-5734	47.174	44.959	44.986	**45.706**	1.2710	3%
Staurosporine	0.001	0.007	0.002	**0.0034**	0.0035	104%
Puromycin	0.693	0.745	0.445	**0.6278**	0.1602	26%

Figure S3: In vitro toxicity of GS-5734 in primary normal human bronchiolar epithelial (NHBE) cell cultures. (A) NHBE cells in 96-wells were incubated with varying concentrations of GS-5734 or two known toxic positive control compounds (puromycin or staurosporin) in triplicate for 48hr after which toxicity was measured by CellTiter-Glo assay. Representative data from three independent studies is shown in Panel A. (**B)** Summary statistics and CC_{50} values for each independent experimental run.

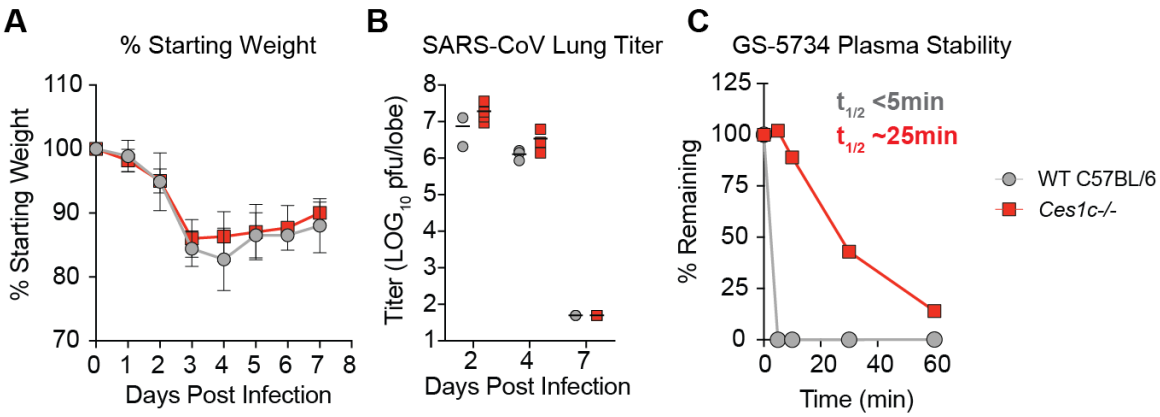


Figure S4: SARS-CoV in vivo pathogenesis is similar in WT and *Ces1c*$^{-/-}$ mice. (A) Percent starting weight of 20 week old female WT C57BL/6 (N =8) and *Ces1c*$^{-/-}$ (N = 11) infected with 10^5 pfu SARS-CoV MA15. **(B)** Virus lung titers as measured by plaque assay. **(C)** GS-5734 plasma stability upon mixing of 1μM GS-5734 with serum from WT and *Ces1c*$^{-/-}$ mice. Half-life was determined in longitudinal samples through measurement of compound via mass spectrometry. All primary data is provided in SM file "Primary Data."

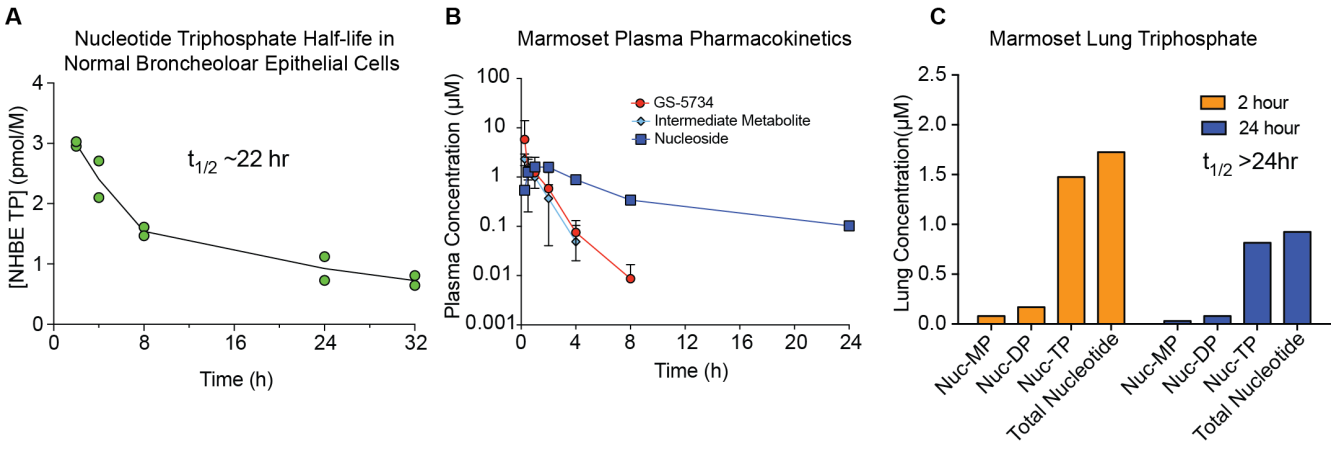


Figure S5: Metabolism in normal human bronchiolar epithelial (NHBE) cells and pharmacokinetic analysis in non-human primates. **(A)** Half-life of nucleotide triphosphate (TP) in NHBE cells (0.2 X 10^6 cells/well in 12-well plates) incubated with 1 µM GS-5734 for 2hr, after which the compound-containing media was removed, cells were washed twice and incubated with fresh medium at 37˚C. TP was measured in longitudinal samples via LC/MS. The TP showed a half-life of approximately 22hr (Panel A). (**B)** The plasma pharmacokinetics in nonhuman primates (marmoset, N = 3) following a single 10 mg/kg IV (slow bolus) dose. (**C)** The kinetics of triphosphate (TP) accumulation in marmoset lung as measured by LC/MS.

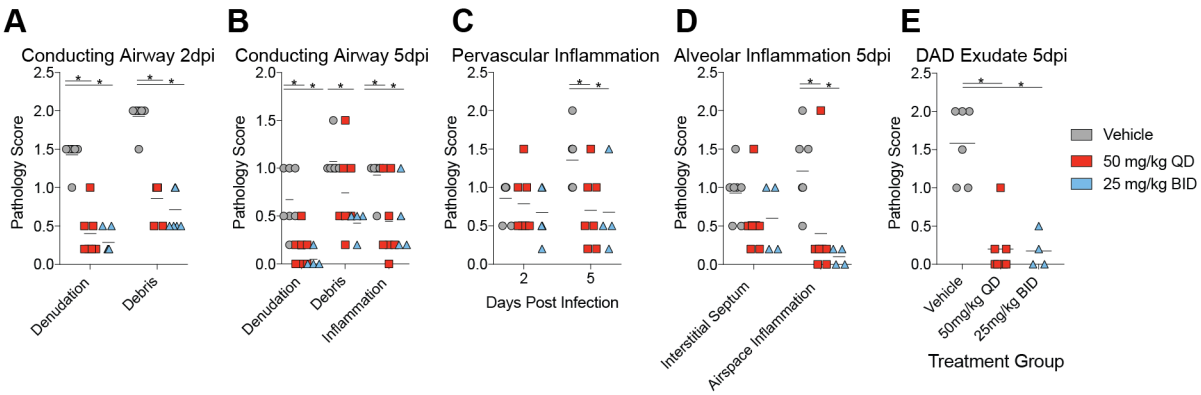


Figure S6: GS-5734 diminishes SARS-CoV induced lung pathology. Mice were infected with 10^4 pfu SARS-CoV MA15 and prophylactically treated with GS-5734 as described in Figure 5. Hematoxylin and eosin stained lung tissue sections were scored blindly for the following SARS-CoV lung pathology metrics. (**A and B)** Early phase damage of conducting airway via denudation of the airway epithelium and accumulation of debris. **(C and D)** Late phase accumulation of inflammatory infiltrates in the perivascular and alveolar space. **(E)** Acute respiratory distress syndrome diffuse alveolar damage exudate 5dpi. Statistical significance was determined using Graphpad Prism Two-way ANOVA with Tukey's multiple comparisons test with a p value cutoff of 0.05. N = 7 mice/group.

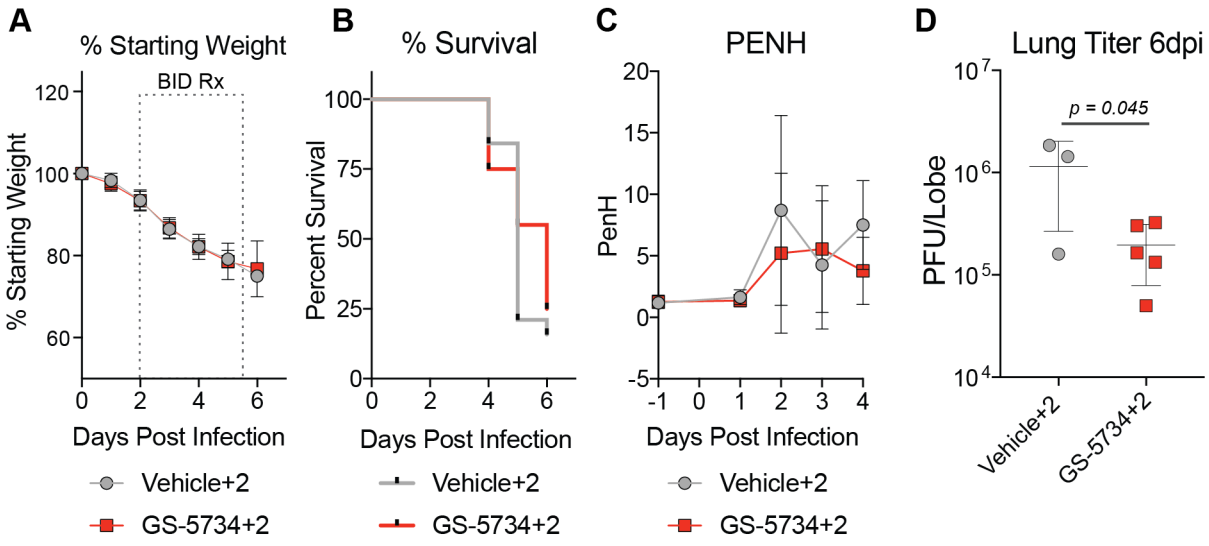


Figure S7: Therapeutic administration of GS-5734 beginning 2dpi does not provide a therapeutic benefit. (**A)** Percent starting weight of 25-27 week old female *Ces1c*$^{-/-}$ infected with 10^3 pfu SARS-CoV MA15 treated twice daily with vehicle or 25mg/kg GS-5734 beginning on +2dpi (vehicle N = 19, GS-5734 N = 20). (**B)** Kaplan-Meyer survival analysis. (**C)** Whole-body plethysmography (WBP) to measure pulmonary function (N = 6/group). Penh is a surrogate measure of bronchoconstriction or airway obstruction and is shown as example data for this study. (**D)** Virus lung titer as measured by plaque assay. Vehicle N=3, GS-5734 treated N = 5. Statistical significance was determined using Graphpad Prism by student's two tailed t-test.

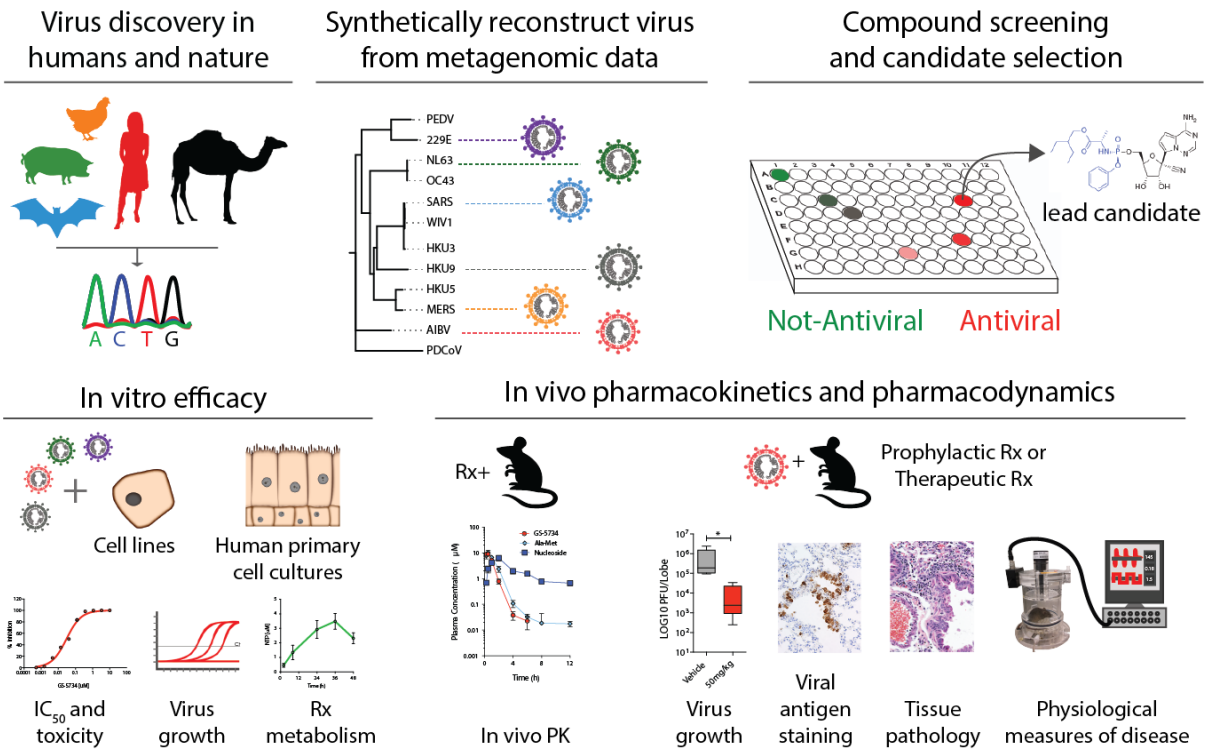


Figure S8: A comprehensive platform approach to evaluate therapeutics against emerging viral infections. Coronaviruses found in human and wild animal populations have remarkable genetic diversity making it difficult to predict which strain may emerge in humans. Thus, broad-spectrum therapeutic approaches are needed to protect against ongoing epidemics and future zoonotic emergence. A platform approach to identify broad-spectrum therapeutics against diverse virus families using in vitro cell culture systems and animal models of disease is described below. Coupling metagenomic data from zoonotic viruses with synthetic genomics, recombinant viral strains that encompass family-wide genetic diversity can be reconstructed and studied. Through compound screening in cell-based antiviral assays and optimization through medicinal chemistry, a lead candidate is selected and rigorously assessed for antiviral activity and metabolism in the most biologically relevant in vitro systems including human primary culture systems. Lastly, lead compound efficacy is assessed in vivo using models of viral pathogenesis reflective of human disease. Importantly, virological, pathological and physiological measures should ideally be assessed to determine the effect of the drug on the virus life cycle and disease symptoms. This process can potentially be applied to any emerging viral pathogen for which such an experimental platform is available or can be built.

Table S1. Coronavirus genomic and subgenomic real-time primer sets

Primer Name	Primer Sequence (5'-3')
MERS Leader Forward	GAATAGCTTGGCTATCTCAC
MERS ORF1 Reverse	CACAATCCCACCAGACAA
MERS ORFN Reverse	TTGTTATCGGCAAAGGAAAC
SARS Leader Forward	AGCCAACCAACCTCGATCTCTTGT
SARS ORF1 Reverse	TGACACCAAGAACAAGGCTCTCCA
SARS ORFN Reverse	ATTGGTGTTGATTGGAACGCCCTG
HKU5 Forward	CTCTCTCTCGTTCTCTTGCAGAAC
HKU5 ORF1 Reverse	GTTGAGCTCTGCTCTATACTTGCC
HKU5 ORFN Reverse	GAATTGGCATTAAGAGGTACGCCC
WIV-1 Forward	GATCTCTTGTAGATCTGTTCT
WIV-1 ORF1 Reverse	GAGTTACTCGTTTCTTGTCAAC
WIV-1 ORFN Reverse	CGTCCTCCATTCTGGTTATT
SHC014 Forward	AGCCAACCAACCTCGATCTCTTGT
SHC014 ORF1 Reverse	AGTTACTCGTTTCTTGTCAACGACAG
SHC014 ORFN Reverse	TCTGTGGGTCCACCAAATGTAATG
HKU3 Forward	GCCAACCAACCTTGATCTCTT
HKU3 ORF1 Reverse	TATACTGCGTAGGTGCGCTA
HKU3 ORFN Reverse	GCGGGTCCACCAAATGTAAT
hGAPDH Forward*	AGCCACATCGCTGAGACA
hGAPDH Reverse*	GCCCAATACGACCAAATCC

* The housekeeping gene hGAPDH was used for normalization of real-time results.

Table S2. Primer/probe sets for indicators of cellular apoptosis/toxicity qRT-PCR

Primer/Probe Target	Assay Reference Number*	Pro or anti Apoptosis?
Bax	Hs00180269_m1	Pro
Bad	Hs00188930_m1	Pro
Bcl2L11	Hs00708019_s1	Pro
Bcl2	Hs00608023_m1	Anti
Mcl1	Hs01050896_m1	Anti
Tradd	Hs00601065_g1	Pro
Fas	Hs00236330_m1	Pro
Tank	Hs00370305_m1	Pro
18S	4352930E	House keeping
GAPDH**	4352934E	House keeping

* Validated assays available from Life Technologies

** The housekeeping gene hGAPDH was used for normalization of real-time results.